ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR DRUG SUBSTANCE, THEIR IMPURITIES AND METABOLITES IN ACTIVE PHARMACEUTICAL INGREDIENTS AND BIOLOGICAL FLUIDS

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By

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Title of the Thesis	:	ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR DRUG SUBSTANCE, THEIR IMPURITIES AND METABOLITES IN ACTIVE PHARMACEUTICAL INGREDIENTS AND BIOLOGICAL FLUIDS
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Analytical Method Development and Validation for Drug Substance, Their Impurities and Metabolites in Active Pharmaceutical Ingredients and Biological Fluids

It is a requisite that the drug substances and products contain specified amounts of active ingredients to ensure that they are at sufficient level to be effective and do not exceed safety limits. Often the drug substances contain impurities arising from various processes which are to be maintained under the specified limits according to their toxicological relevance.

Drug products get exposed to various environmental and other conditions during their storage and their stability with respect to active ingredient content, change in levels of impurities, formation of any new impurities (due to degradation or other reactions with the container closer systems) needs to be monitored.

During safety and efficacy assessment of the drug substances, it is required to monitor their content in biological matrices. Drug substances also undergo various reactions in the biological systems and form metabolites. Hence, along with active ingredient content metabolite contents also need to be monitored in biological matrices.

Reliable and sufficiently sensitive analytical methods play a key role in all these monitoring requirements. Various types of analysis require different types of analytical techniques.

Impurities in Active Pharmaceutical Ingredients (API) and Active Ingredients (AI) have gained lot of public and media attention in the recent past due to the possible activity of the impurity to turn out as a potential toxic substance even when present in small quantities. As per the ICH guidelines, the presence of unknow impurities should be controlled less than 0.1% (1000 ppm) and some specific impurities falling in the category of structural allerts should be controlled in the range of less than 6 ppm or as specificed.

This calls for the development of sensitive methods using finer tools like High Pressure Liquid Chromatography (HPLC), Liquid Chromatography Mass Spectroscopy (LC-MS/MS), Gas Chromatography (GC), etc to identify and / or qualify the impurities at a

very low level of their presence in addition to reliably analysing the active ingredient content.

To ensure that the developed method is sound and relieable enough, the methods are generally subjected to validation covering parameters like specificity, precision, limit of quantitation, limit of detection, linearity, range and accuracy, as applicable.

In this work, an effort has been put in to develop and validate analytical methods to evaluate impurities and metabolites in Active Pharmaceutical Ingredient, Oral Dosage Form, and biological fluids.

- A new reverse phase method, which is stability indicating, was developed, and validated to simultaneously determine rosuvastatin and its lactone impurity in rosuvastatin calcium.
- A new chiral method was developed and validated for separation, identification, and quantification of enantiomer of rosuvastatin in rosuvastatin calcium.
- To evaluate Class I and 2A elemental impurities in rosuvastatin tablets, an ICPMS method was developed and validated.
- LC-MS/MS method was developed and validated to evaluate 8 nitrosamines in rosuvastatin calcium.
- LC-MS/MS method was developed and validated to evaluate rosuvastatin and its metabolite in human plasm, dog plasma, rat plasma and rat urine.
- Structure elucidation was performed for the process related impurities in azimsulfuran technical material and a method for isolation and generation of some of the identified impurities and chromatographic separation technique for analytical separation and analysis of the impurities were developed.

In summary, the thesis is divided into following eight chapters.

Chapter 1: General Introduction

This is an introductory chapter which gives a brief review on the impurities, classification of impurities, likely consequences of their presence in the APIs (Active Pharmaceutical Ingredients) and Drug products. Importance of analyzing impurity content along with active ingredient content using validated analytical methods that facilitate analysis at

lowest possible concentration levels are discussed. Various regulations governing the impurity content and also the requirements of the analytical methods used for analysis are reviewed. This chapter also gives details on used analytical techniques and importance of method validation and its parameters.

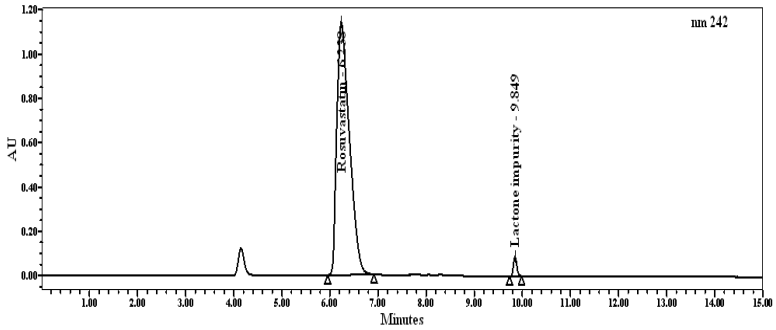
Chapter 2: Characterisation of Rosuvastatin Calcium

This chapter reports the characterization of Rosuvastatin calcium standard using the spectra of FTIR, Proton NMR, ^{13}C NMR, 2D-COSY, and 2D-HSQC and HR-MS. Purity confirmation of the standard is done by UHPLC. The structure of rosuvastatin calcium standard is confirmed through analysis and interpretation of spectral and analytical data.

Sample was mixed with potassium bromide and IR spectrum was recorded. The characteristic bands obtained at the wave numbers specific for the functional group confirm the structure of rosuvastatin. Mass spectrometry experiments were performed using TOF (time of flight) mass analyser. The molecular ion peak in HR-MS is of m/z 482.1712 (M+H) and the spectra confirm to the molecular weight (481.16) correspond to rosuvastatin. NMR spectroscopy experiments were performed in DMSO-d6 as solvent.
The ^{1}H-NMR, ^{13}C-NMR, 2D-COSY, and 2D-HSQC peaks of the spectra were identified. The chemical shifts values are consistent with the molecular structure for rosuvastatin. Reverse phase UHPLC analysis with UV detection at 254 nm was carried out to assess the purity of rosuvastatin standard. The purity of the rosuvastatin by UHPLC is 99.76%.

Chapter 3: Development and Validation of Stability Indicating Analytical Method by Reverse Phase HPLC for Rosuvastatin and its Lactone Impurity in Rosuvastatin Calcium API and Drug Product

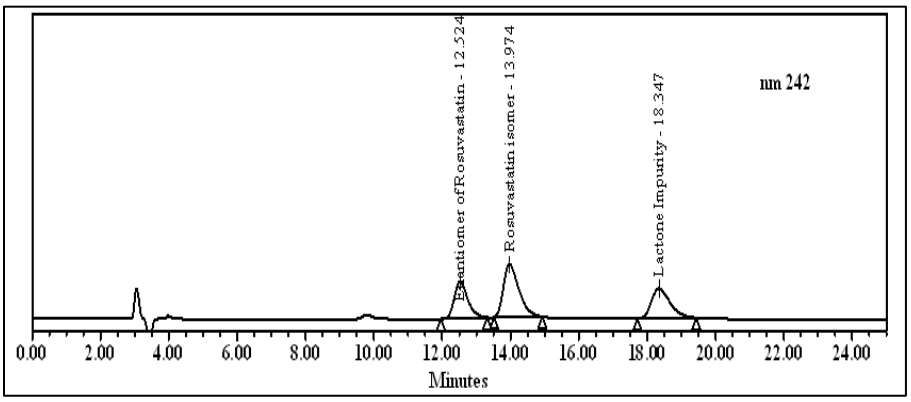
A simple, specific, linear, accurate, precise and sensitive reverse phase HPLC method was successfully developed and validated for simultaneous quantification of lactone impurity and rosuvastatin by using a mobile phase containing ammonium acetate, acetonitrile and methanol, which is capable of separating the rosuvastatin calcium and its lactone impurity. This chapter describes this novel reverse phase HPLC method for analysis of rosuvastatin Calcium and its lactone impurity in drug substance (API) and pharmaceutical dosage form. This is a stability indicating method which was studied under various degradation stress conditions.



The chapter also describes the validation parameters of the method like Specificity, Precision, Limit of Detection, Limit of Quantitation, Linearity and Range. The obtained results of the experiment are discussed in this chapter.

Chapter 4: Development and Validation of Chiral HPLC Method for Separation and Quantification of Enantiomer in Rosuvastatin Calcium API

This chapter deals with simple, precise, rapid and accurate normal phase enantioselective high performance liquid chromatographic method which was developed for enantiomeric resolution of rosuvastatin. Cellulose tris (3,5-dimethylphenylcarbamate) based chiral column CHIRALPAK IB is found to be selective for the separation of enantiomer of rosuvastatin calcium. This novel normal phase chiral HPLC method is useful for quantitation of enantiomer in the Rosuvastatin Calcium.



The detailed method and the method validation parameters like Specificity, Precision, Limit of Detection, Limit of Quantitation and Linearity and Range; and the results obtained are discussed in this chapter.

Chapter 5: Development and Validation of ICPMS Method for Quantification of Elemental Impurities in Rosuvastatin Calcium Drug Product

This chapter gives details on an ICPMS method used in determining the Class I and Class IIA elemental impurities in Rosuvastatin formulation. Elemental impurities are classified as Class I, Class II (IIa and 2B) and Class III based on its toxicity. It is a mandatory regulatory requirement currently to test all the drug substance for Class I and Class IIA elemental impurities which needs to be controlled within specified limits based on daily dosage.

In our current work we have developed a sample digestion method that do not use hydrofluoric acid and also does not require sample evaporation in graphite furnace after microwave digestion. This method is simple to operate and reproducible meeting the regulatory requirements with respect to quantitation limits.

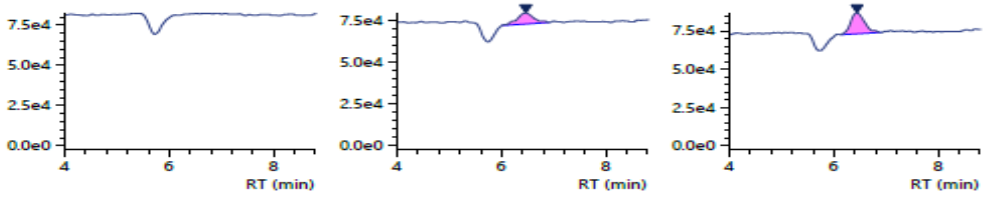
Achieved quantitation limits		
Element	LOD (ppb)	LOQ (ppb)
Cd	0.04	0.125
Pb	0.04	0.125
As	0.12	0.375
Hg	0.25	0.750
Co	0.41	1.25
V	0.83	2.50
Ni	1.65	5.00

The method validation parameters like Specificity, Precision, Limit of Detection, Limit of Quantitation and Linearity and range and the findings shall be discussed in this chapter.

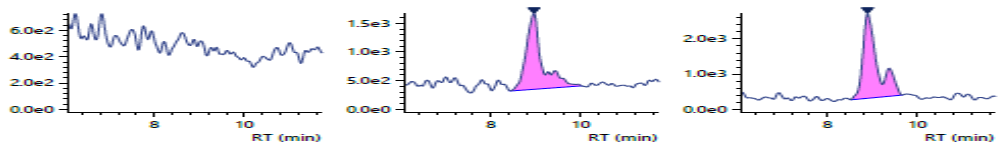
Based on the results evaluated from validation parameters, all validation parameters meet the acceptance criteria. Hence, the given analytical method involving microwave digestion of the drug product (tablets) in acid mixture followed by analysis using ICP-MS equipment is deemed to be fit for the quantitative determination of elemental impurities, Cd, Pb, As, Hg, Co, V and Ni in rosuvastatin tablets.

Chapter 6: Development and Validation of LC-MS/MS Method For Quantification of N-Nitrosamine Impurities in Rosuvastatin Calcium Drug Product

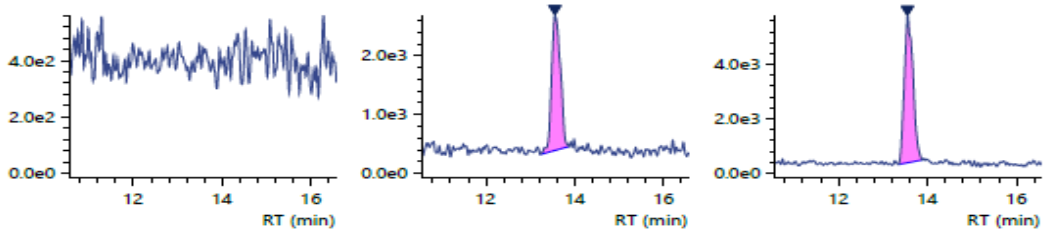
This chapter describes the LC-MS/MS method to determine Nitrosamine impurities in Rosuvastatin Calcium drug substance. Nitrosamines have gained momentum in the pharmaceutical industry and has become essential substances to be controlled at a very low limits due to their carcinogenic properties. LC-MS/MS method for analysis of N-nitroso-dimethylamine (NDMA), N-nitroso-n-methyl-4-aminobutyric acid (NMBA), N-nitroso-diethylamine (NDEA), N-nitroso-ethylisopropylamine (NEIPA), N-nitroso-diisopropylamine (NDIPA), N-nitroso-dipropylamine (NDPA), N-nitroso-methyl phenylamine (NMPA) and N-nitroso-di-n-butylamine (NDBA) in rosuvastatin calcium tablet is developed and validated.



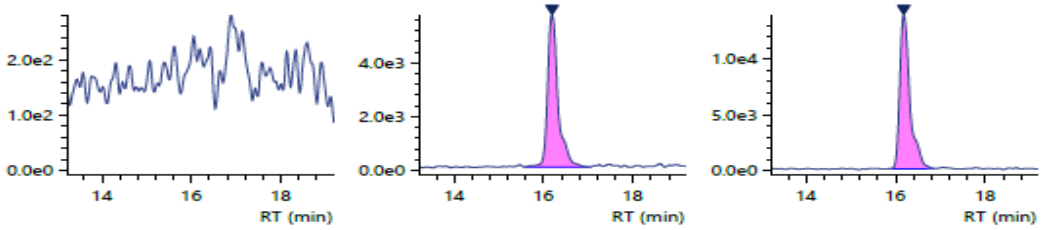
Typical NDMA blank, LOD and LOQ level LC-MS/MS chromatogram



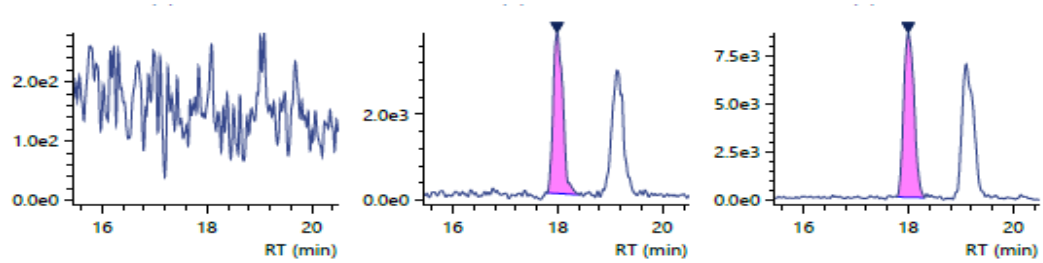
Typical NMBA blank, LOD and LOQ level LC-MS/MS chromatogram



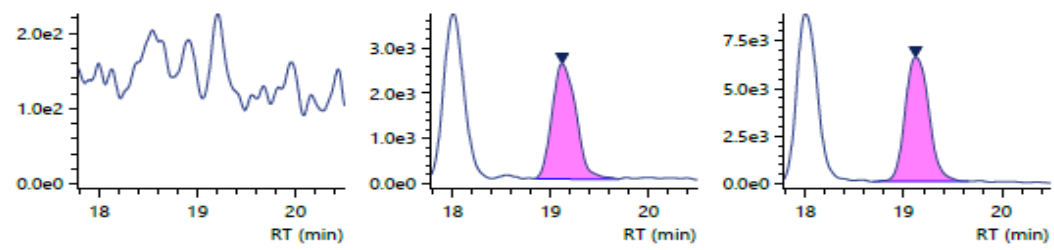
Typical NDEA blank, LOD and LOQ level LC-MS/MS chromatogram



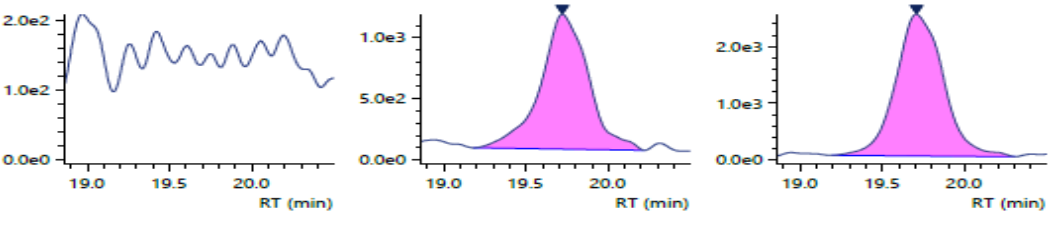
Typical NEIPA blank, LOD and LOQ level LC-MS/MS chromatogram



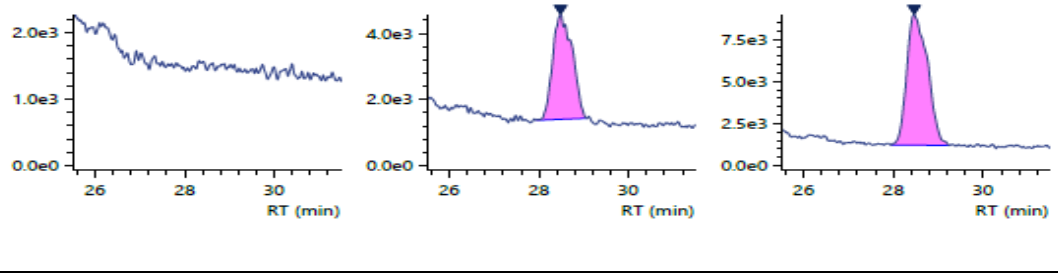
Typical NDIPA blank, LOD and LOQ level LC-MS/MS chromatogram



Typical NDPA blank, LOD and LOQ level LC-MS/MS chromatogram



Typical NMPA blank, LOD and LOQ level LC-MS/MS chromatogram



Typical NDBA blank, LOD and LOQ level LC-MS/MS chromatogram

The developed method was successfully validated, and results indicate that the method is precise, linear and accurate with adequate sensitivity at ppb levels. The validated method is suitable for the analysis of listed nitrosamines in the rosuvastatin tablet.

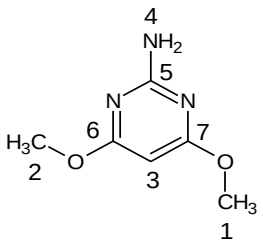
Chapter 7: Development and Validation of LC-MS/MS Method for Quantification of Rosuvastatin and its Metabolite in Human Plasma, Dog Plasma, Rat Plasma and Rat Urine

Bioanalysis is the quantitative determination of drugs and their metabolites in biological fluids. This technique is used very early in the drug development process to provide support to drug discovery programs on the metabolic fate and pharmacokinetics of chemicals in living cells and in animals. Its use continues throughout the preclinical and clinical drug development phases, into post-marketing support and may sometimes extend into clinical therapeutic drug monitoring.

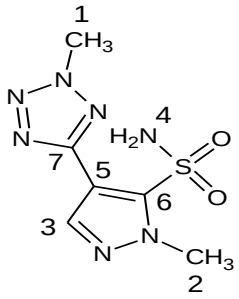
In this chapter simple, robust LC–MS/MS method is described for quantitative determination of rosuvastatin and its metabolite in various biological matrices like human plasma, dog plasma, rat plasma and rat urine. These methods involve simple protein precipitation followed by LC-MS/MS analysis. These developed bioanalytical methods were successfully validated for analysis of rosuvastatin and its metabolite in human plasma, dog plasma, rat plasma and rat urine. The validation results indicate the suitability of the developed bioanalytical methods for analysis of rosuvastatin and its metabolite in the tested biological matrices. Consistency of these validation results across various matrices further indicate that these methods can be used for various other matrices as well The detailed method and method validation parameters like Linearity and Range; specificity, precision, Limit of Quantitation and Accuracy are discussed in this chapter.

Chapter 8: Method Development, Isolation, Characterization and Quantification of Process Related Impurities in Azimsulfuron Technical

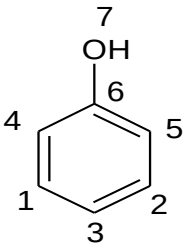
This chapter gives details on identification, characterization and quantification of process related impurities in Azimsulfuron technical. This also chapter describes isolation and characterization of Azimsulfuron impurities. Six impurities ranging from 0.05 to 0.15 % w/w in Azimsulfuron technical were detected by a simple reverse phase HPLC method. These impurities were isolated by prep-HPLC and were characterized using their IR, ^{1}H NMR, ^{13}C NMR and LC-MS/MS spectral data. The detailed results are discussed in this chapter.



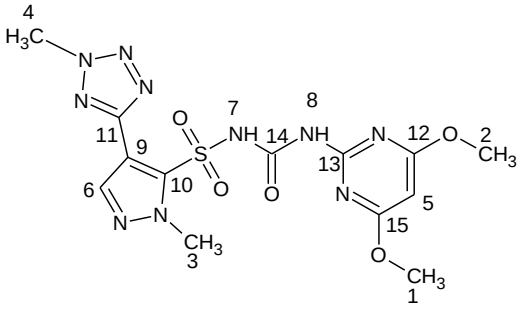
Impurity A



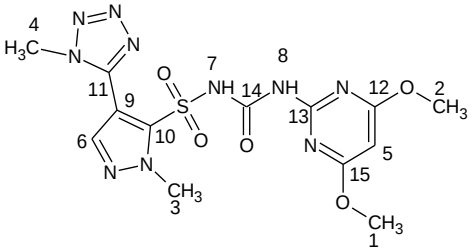
Impurity B



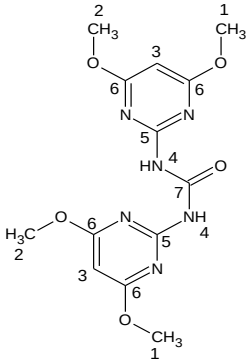
Impurity C



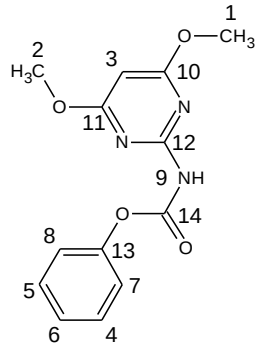
Azimsulfuron



Impurity D



Impurity E



Impurity F

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LIST OF RESEARCH ARTICLES PUBLISHED AND PATENTS

1. **A New Simultaneous Determination of Rosuvastatin Calcium and its Lactone Impurity by Reverse Phase HPLC method**

   Moodbidri Prabhu Venkatesh, Dhayanithi Varadaraji, Manjunathashastry Ganesh Belavadi, Pati Hari Narayan and Vasireddy Pardhasaradhi

   Asian J. Pharm. Res. 2015; Vol. 5, Issue 4, Page 175-182

2. **Development and Validation of Chiral HPLC method for Quantitation of Enantiomers in Rosuvastatin Calcium**

   Prabhu Venkatesh Moodbidri, Varadaraji Dhayanithi, Ganesh Belavadi Manjunatha shastry, Hari Narayan Pati and Pardhasaradhi Vasireddy

   International Journal of Pharmaceutics & Drug Analysis 2015; Vol. 3, Issue 9, Page 275 – 281

3. **Identification, Charecterization and Quantification of Process Related Impurities in Azimsulfuron Technical**

   M. Parbhu Venkatesh, V. Pardhasaradhi, V. Dhayanithi, Mane Avinash, S. Palimkar, R. Kadam Subhash and Hindupur Rama Mohan

   Chemical Science Transactions, Volume 5, Issue 1, Page 41-55

4. **Identification and synthesis of major impurities formed during synthesis of trifloxystrobin and kresoxim-methyl fungicides.**
   Pasumpon K, Antao N, Singh A, SatamVS, Kadam S, Prabhu VM, Hindupur RM, Pati HN.

   Letters in Organic Chemistry 2015;12: 306-310.

5. **United Kingdom Patent Application No. 1215177.5:**

Filed on: 24 Aug 2012; claiming Priority from Indian Patent Application No. 2535/CHE/2012; Priority date: 26 June 2012.
Title: "Substituted Pyrazole Compounds and Process Thereof" Also applied for PCT: PCT/IN2013/000250

6. **United Kingdom Patent Application No. 1215178.3:**
Filed on: 24 Aug 2012; Claiming Priority from Indian Patent Application No. 2536/CHE/2012; Priority date: 26 June 2012.
Title: "Substituted Tetrazole Compounds and Process Thereof" Also applied for PCT: PCT/IN2013/000251

7. **United Kingdom Patent Application No. 1215176.7:**
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Title: "Process of preparation of Azimsulfuron" Also applied for PCT: PCT/IN2013/000252

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